VALIDATED LIQUID CHROMATOGRAPHIC METHOD FOR THE ESTIMATION OF ANTIHYPERTENSIVE MIXTURE IN PHARMACEUTICAL DOSAGE FORMS

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ABSTRACT
A rapid and precise RP-HPLC method for determination of Hydralazine and Hydrochlorothiazide in bulk and pharmaceutical dosage forms. Hydralazine & Hydrochlorothiazide are found to be degraded together under different set of conditions as followed according to ICH guidelines and the degradants so formed along with Hydralazine & hydrochlorothiazide are separated by using INERTSIL ODS C18 (150 x 4.6, 5µ) using mobile phase 0.01M potassium dihydrogen orthophosphate buffer, pH was adjusted to 4.8 with ortho phosphoric acid and acetonitrile (60:40) with a flow rate of 1ml/min, with a detection wavelength of 217nm for both the compounds with a injection volume of 20µl. The method was validated for selectivity, linearity, accuracy, robustness, precision and specificity. The results were indicating the method was selective in analysis of both Hydralazine and hydrochlorothiazide in the presence of degradation products formed under various stress conditions.

Keywords: Hydralazine, Hydrochlorothiazide, INERTSIL ODS C18, Validation Stability.
INTRODUCTION
Hydralazine chemically it is 1-hydrazinylphthalazine is a direct acting smooth muscle relaxant used to treat hypertension by acting as a vasodilator primarily in arteries and arterioles. Vasodilators act to decrease peripheral resistance, thereby lowering blood pressure and decreasing afterload. It has also clinical application in after heart valve replacement and in the treatment of chronic – resistant heart failure\textsuperscript{[1, 2]}. It is widely used in combination with β-blocking drug (to balance the reflex tachycardia) and a diuretic (to decrease sodium retention) for the treatment of essential hypertension. HDZ increases cyclic guanosine monophosphate (cGMP) levels, increasing the activity of protein kinase G (PKG). Active PKG adds an inhibitory phosphate to myosin light-chain kinase (MLCK) - a protein involved in the activation of cross-bridge cycling (i.e. contraction) in smooth muscle. This results in blood vessel relaxation\textsuperscript{[3, 4]}. Hydralazine derivatives and their formulations are official in British Pharmacopoeia (BP), Indian Pharmacopoeia (IP) and United States Pharmacopoeia (USP)\textsuperscript{[5-7]}. Hydrochlorothiazide is 6-Chloro-3, 4-dihydro-2H-1, 2, 4-benzothiadiazine-7-sulfonamide 1, 1-dioxide. It reduces the amount of water in the body by increasing the flow of urine, which helps lower the blood pressure\textsuperscript{[8]}. Hydralazine and Hydrochlorothiazide are introduced into the market in combined dosage form, which is widely used in the treatment of hypertension Literature review reveals that the methods for Hydralazine and hydrochlorothiazide alone or in combined dosage forms , a wide variety of analytical methods have been reported for the determination of Hydralazine in pharmaceutical preparations and in biological fluids which includes Colorimetry\textsuperscript{[9]}, Spectrophotometry\textsuperscript{[10,11]}, Spectrofluorometry\textsuperscript{[12]}, Gas Chromatography\textsuperscript{[13-17]}, Gas chromatography–Mass Spectroscopy(GC-MS)\textsuperscript{[18]}, High Performance Liquid Chromatography-Ultra violet Visible Spectroscopy (HPLC-UV)\textsuperscript{[19-22]}, High Performance Liquid Chromatography – Electrochemical Detection\textsuperscript{[23]} and Liquid Chromatography - Electron Spray Ionization - Tandem Mass Spectrometry (LC-ESI-MS/MS)\textsuperscript{[24]}.There are several reports of the determination of Hydrochlorothiazide alone\textsuperscript{[25]} or in combination with other ARA-II drugs including Spectrophotometry\textsuperscript{[26-28]}, HPLC\textsuperscript{[29-42]}, HPTLC-densitometry\textsuperscript{[43-46]}, Liquid Chromatography- Tandem Mass Spectrometry (LC-MS/MS)\textsuperscript{[47, 48]}, capillary electrophoresis and capillary electro chromatography\textsuperscript{[49, 50]}. Based on the literature review, there is no liquid chromatographic method for the simultaneous determination of hydralazine and hydrochlorothiazide in bulk and pharmaceutical dosage forms, the aim of the study to develop the validated chromatographic procedure for their determination.
Experimental

I Chemicals and Reagents

Hydralazine & Hydrochlorothiazide reference standards was supplied by Solvey pharmaceuticals limited (Hyderabad, India). Potassium dihydrogen ortho phosphate, Acetonitrile, Methanol, Water all of HPLC grade, orthophosphoric acid was purchased from Merck (Mumbai, India). All chemicals were of analytical grade.

II Chromatography

Based on the method development trials indicated in Table 1, the optimized condition for determination was carried out on Waters HPLC 2695 equipped with PDA 2487 as detector using data handling system – waters empower 2.0 software. The column used in the development for the determination is INERTSIL ODS C18 (150 x 4.6, 5µ) using a mobile phase of 0.01M potassium dihydrogen orthophosphate buffer, pH was adjusted to 4.8 with ortho phosphoric acid and acetonitrile (60:40). The detector wavelength was set at 217 nm for both the components. A flow rate of 1 ml/min was used for the determination of Hydralazine and hydrochlorothiazide. Mobile phase acts as diluent and 20µL sample were injected into HPLC system at the column and sample temperature of 30ºc.

III Mobile phase

Accurately weighed 0.68gm of potassium dihydrogen phosphate was mixed with 500mL of HPLC water and pH was adjusted to 4.8 with Orthophosphoric Acid, filtered through 0.45µm Membrane filter. 0.01 M phosphate buffer pH 4.8 and Acetonitrile in the ratio of 60:40 used as a mobile phase was degassed and injected into the system.

Table 1 Method Development conditions

<table>
<thead>
<tr>
<th>Trial</th>
<th>Type of column</th>
<th>Mobile phase composition</th>
<th>Injection volume</th>
<th>Flow</th>
<th>Defect</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C18 (150 x 4.6),5µm</td>
<td>Water : Acetonitrile (30:70)</td>
<td>20µl</td>
<td>1ml/min</td>
<td>Resolution was not so good</td>
</tr>
<tr>
<td>2</td>
<td>C18 (150 x 4.6),5µm</td>
<td>Buffer(pH 4.8): Acetonitrile (50:50)</td>
<td>20µl</td>
<td>1ml/min</td>
<td>More tailing and less resolution</td>
</tr>
<tr>
<td>3</td>
<td>C18 (150 x 4.6),5µm</td>
<td>Buffer (pH 4.8): Acetonitrile (70:30)</td>
<td>20µl</td>
<td>1ml/min</td>
<td>More broad peaks</td>
</tr>
<tr>
<td>4</td>
<td>C18 (150 x 4.6),5µm</td>
<td>Buffer (pH 4.8): Acetonitrile: (40:60)</td>
<td>20µl</td>
<td>1ml/min</td>
<td>More broad peaks</td>
</tr>
<tr>
<td>5</td>
<td>C18 (150 x 4.6),5µm</td>
<td>Buffer (pH 4.8): Acetonitrile: (60:40)</td>
<td>20µl</td>
<td>1ml/min</td>
<td>More theoretical plates, less tailing, Good resolution.</td>
</tr>
</tbody>
</table>
IV Preparation of standard and sample solutions
Accurately weighed and transferred about 25 mg of Hydralazine, 25 mg of Hydrochlorothiazide working standards into two separate 100 ml volumetric flasks, add about 60 ml of diluent and sonicated to dissolve, cool the solution to room temperature & dilute to the volume with diluent. 10 ml of the above standard stock solutions of Hydralazine and Hydrochlorothiazide, was transferred into another two separate 100 ml volumetric flask and dilute to the volume with diluent. Ten tablets were weighed and powdered uniformly in a mortar. An accurately weighed portion powder equivalent to 25mg of Hydralazine was transferred into a 100 ml volumetric flask. 60 ml of diluent was added, sonicated for 10 minutes with occasional stirring. Cool the solution to room temperature and dilute to the volume with diluent, filtered the solution through 0.45µm Teflon filter syringe. 10 ml of the above filtered solution was transferred into a 100 ml volumetric flask & dilute to the volume with diluent. The sample chromatogram was depicted in the figure 1.

![Image](image.png)

**Figure 1 Sample chromatogram for Hydrochlorothiazide and Hydralazine**

Validation
The method was validates in accordance with ICH guidelines.

I Linearity
A calibration curve was made and concentration examined within the detection range of 6.25-38.75µg/ml for both Hydralazine & hydrochlorothiazide and correlation coefficient was found to be 0.999 for both the compounds respectively.

II Precision
The precision (expressed as the relative standard deviation (RSD) was determined for
Hydralazine & hydrochlorothiazide for repeated analysis and the values are presented in Table 2.

Table 2 Precision studies for Hydralazine and Hydrochlorothiazide

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% RSD (Day-1, Analyst-1)</th>
<th>% RSD (Day-2, Analyst-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retention Time</td>
<td>Area</td>
</tr>
<tr>
<td>Hydralazine</td>
<td>0.040</td>
<td>0.06</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>0.046</td>
<td>0.12</td>
</tr>
</tbody>
</table>

III Recovery

The recovery experiment values obtained were performed by adding a fixed amount of drug to preanalysed formulation summarized in Table 3.

Table 3 Recovery studies for Hydralazine and Hydrochlorothiazide

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount added (mg)</th>
<th>Amount recovered (mg)</th>
<th>% recovery</th>
<th>Mean % recovery</th>
<th>% RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydralazine</td>
<td>6.25</td>
<td>6.2068</td>
<td>99.31</td>
<td>99.32</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>12.3825</td>
<td>99.06</td>
<td>99.69</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>18.75</td>
<td>18.6131</td>
<td>99.27</td>
<td>99.51</td>
<td>0.2</td>
</tr>
<tr>
<td>Hydrochlorothiazide</td>
<td>6.25</td>
<td>6.2344</td>
<td>99.75</td>
<td>99.69</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>12.39</td>
<td>99.12</td>
<td>99.51</td>
<td>0.170</td>
</tr>
<tr>
<td></td>
<td>18.75</td>
<td>18.6581</td>
<td>99.51</td>
<td>99.51</td>
<td>0.170</td>
</tr>
</tbody>
</table>

Table 4 Stability studies for Hydralazine and Hydrochlorothiazide

<table>
<thead>
<tr>
<th>Experiment</th>
<th>% Degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid (1N HCL,2ml)</td>
<td>HYDRAZINE 1.15</td>
</tr>
<tr>
<td></td>
<td>HYDROCHLOROTHIAZIDE 5.07</td>
</tr>
<tr>
<td>Alkali (1N NaOH, 2ml)</td>
<td>HYDRAZINE 1.60</td>
</tr>
<tr>
<td></td>
<td>HYDROCHLOROTHIAZIDE 25.44</td>
</tr>
<tr>
<td>Thermal (Heating at 60ºc for 1hr)</td>
<td>HYDRAZINE 35.25</td>
</tr>
<tr>
<td></td>
<td>HYDROCHLOROTHIAZIDE 0.02</td>
</tr>
</tbody>
</table>

IV Stability

The stability of sample was checked by forced degradation in different conditions and % of degradation was calculated. The values in Table 4 indicating that any other impurity is not merging with the main peak (Figure 2-4)
Figure 2 Acid degradation chromatogram of Hydralazine and Hydrochlorothiazide

Figure 3 Basic degradation chromatogram of Hydralazine and Hydrochlorothiazide

Figure 4 Thermal degradation chromatogram of Hydralazine and Hydrochlorothiazide
V Robustness
The reliability of the method was determined by made small deliberate variations in method parameters and the RSD values (Table 5) obtained, an indication of its reliability on normal usage.

Table 5 Robustness studies for Hydralazine and Hydrochlorothiazide

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Conditions</th>
<th>Hydralazine</th>
<th>Hydrochlorothiazide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Retention Time</td>
<td>Area</td>
</tr>
<tr>
<td>1</td>
<td>Flow (+0.2ml)</td>
<td>4.271</td>
<td>5840281</td>
</tr>
<tr>
<td>2</td>
<td>Flow (-0.2ml)</td>
<td>3.278</td>
<td>4457969</td>
</tr>
<tr>
<td>3</td>
<td>Organic (+2 %)</td>
<td>3.613</td>
<td>5109160</td>
</tr>
<tr>
<td>4</td>
<td>Organic (-2 %)</td>
<td>3.518</td>
<td>5096105</td>
</tr>
</tbody>
</table>

RESULTS AND DISCUSSION
The conditions tested for method development indicates that all the system suitability parameters according to ICH guidelines was achieved by using INERTSIL ODS C18 (150 x 4.6, 5µ) column using mobile phase using mobile phase 0.01M potassium dihydrogen orthophosphate buffer, pH was adjusted to 4.8 with ortho phosphoric acid and acetonitrile (60:40) with a flow rate of 1ml/min, with a detection wavelength of 217nm for both the compounds with a injection volume of 20µl. The assay values obtained by proposed method for Hydrochlorothiazide and Hydralazine present in the tablets were found to be 99.56% and 99.57% respectively. The LOD values for Hydralazine and Hydrochlorothiazide were 0.3983 µg/ml and 0.4601 µg/ml, while LOQ values were 1.2071 µg/ml and 1.3942 µg/ml respectively.

CONCLUSION
A method was developed for the determination of Hydralazine & hydrochlorothiazide in tablets which is rapid, stable & specific. The results indicate that the described method can be used for quantitative analysis of the compounds.
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